Pestic. Sci. 1998, **54**, 43–51

Comparative Effects of the Herbicide Dinitro-*o*-cresol on Mitochondrial Bioenergetics

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(Received 15 January 1998; revised version received 15 April 1998; accepted 5 May 1998)

Abstract: The herbicide dinitro-*o*-cresol (DNOC) was evaluated for its effects on bioenergetic activities of potato tuber mitochondria to elucidate its mechanism of action and to compare its toxicological properties with those of the chemically related uncoupler dinitrophenol (DNP). DNOC acts as a typical uncoupler, similarly to the classical uncouplers DNP and carbonylcyanide *p*-trifluoromethoxyphenyl-hydrazone (FCCP). Low concentrations of DNOC (<100 μM) maximally stimulate succinate-supported respiration of plant mitochondria, with simultaneous collapse of trans-membrane electrical potential, more efficiently than DNP. The herbicide makes the plant mitochondrial membrane more permeable to protons, acting as a protonophore even in non-energized mitochondria. High concentrations of DNOC (>100 μM) act also more efficiently than DNP simultaneously as a protonophore and inhibitor of respiration, especially when respiration is supported by substrates that are transported to the matrix. The efficiency of DNOC is decreased with increase of mitochondrial protein, BSA and exogenous orthophosphate. Although similar effects were observed for animal and plant mitochondria, rat-liver mitochondrial respiration was more sensitive to DNOC than plant mitochondria. Furthermore, in the presence of DNOC, liver mitochondria exhibited a higher state 3 respiratory coupling level than potato tuber mitochondria, as a result of a considerable stimulation (60%) of state 3 respiration. In conclusion, DNOC is a more potent mitochondrial uncoupler and respiratory chain inhibitor than DNP, although their chemical structures are very similar. Apparently, the additional methyl group of DNOC increases its efficiency as an uncoupler and as an inhibitor, as compared to DNP. Plant mitochondria were shown to be as useful as animal mitochondria in evaluating the toxicity of these xenobiotics. © 1998 Society of Chemical Industry.

Pestic. Sci., **54**, 43–51 (1998)

Key words: herbicide; mitochondria; *Solanum tuberosum*; dinitro-*o*-cresol

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Contract/grant sponsor: PBIC, Portugal; Contract/grant number: PBIC/C/BIO 1985/95.
Contract/grant sponsor: Praxis, Portugal; Contract/grant number: Praxis/2/2.1/BIO/1156/94.
Contract/grant sponsor: PADCT, Brazil; Contract/grant number: Proc. 620123/94-0.
Contract/grant sponsor: Convénio Junta Nacional de Investigação Científica of Portugal (JNICT); Contract/grant number: Proc. no. 423/FAPESP.
Contract/grant sponsor: Fundação de Amparo à Pesquisà do Estado de São Paulo of Brazil (FAPESP). Contract/grant number: Proc. no. 1996/0739-9.

 Pestic. Sci. 0031-613X/98/$17.50. Printed in Great Britain

1 INTRODUCTION

In spite of considerable but unfocused information,[1–7] there are few detailed data on the effects of herbicides on mitochondrial metabolism. Plant mitochondria themselves have been used very sparingly.[8–11] These organelles provide the eukaryotic cell of non-photosynthetic tissue with more than 90% of its total energy requirements. Suspensions of plant mitochondria can be obtained with a high degree of purity and intactness[12] and changes in activity can be studied by several methods: ion permeability, swelling, polarography, evaluation of trans-membrane potentials and enzyme activities. Several herbicides interact with mitochondria, being classified, according to their effects, as uncoupling agents, electron-transport inhibitors, or energy-transfer inhibitors.[13]

There are few detailed studies on the effects of dinitro-*o*-cresol (DNOC; 2-methyl-4,6-dinitrophenol) on mitochondria reported in the literature.[13] Braunbeck and Völkl[14] used European eel (*Anguilla anguilla* L.), a fish not particularly sensitive to xenobiotic compounds. The results revealed effects on a few mitochondrial enzymatic activities and some ultrastructural changes. DNOC mimics the physiological effects of uncouplers by stimulating the respiration of humans, insects, yeast and higher plant mitochondria,[15,16] but there are no studies on its mechanism of toxicity compared to classical uncouplers, such as 4,6-dinitrophenol (DNP).

DNOC has been studied for its bioenergetic toxicity interactions in comparison to DNP,[17] because of the similarities of their chemical structures (Fig. 1). The potential use of plant instead of animal mitochondria in the toxicological screening of xenobiotics of industrial and agrochemical interest has also been evaluated by carrying out studies with DNOC in potato tuber mitochondria and rat liver mitochondria, two standard biological materials used as representatives from plant and animal sources.

2 METHODS

2.1 Preparation of mitochondria

Fresh potato tubers (*Solanum tuberosum* L.) were obtained from the local market. Mitochondria were isolated and purified according to a procedure involving Percoll gradient centrifugation as a terminal purification step,[12] except that 22% Percoll instead of 28% was used. Furthermore, phenylmethylsulphonyl fluoride (500 μM) + dithiothreitol (2 mM) were used as protective agents, instead of cysteine. These modifications preserved the integrity and activities of isolated mitochondria for more than 24 h. The mitochondrial fraction was collected from the Percoll gradient with a Pasteur pipette and washed twice, by centrifugation at 30 000*g* for 5 min in washing medium (medium A) containing mannitol (300 mM) bovine serum albumin (BSA; 1 g litre^{-1}) and Hepes (pH 7·2; 10 mM). The pellet was gently resuspended in medium A at a protein concentration of 20–30 mg ml^{-1}. Rat liver mitochondria were obtained by differential centrifugation.[7] Protein was determined by the procedure of Bradford.[18]

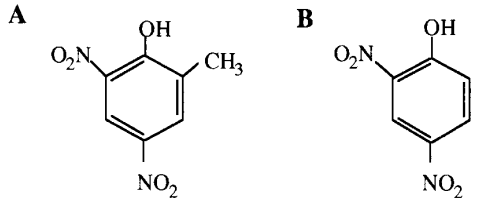

Fig. 1. Structure of (A) dinitro-*o*-cresol and (B) dinitrophenol.

2.2 Measurement of respiratory activities

Oxygen consumption was monitored with a Clark oxygen electrode at 25°C. The polarographic measurements were performed in 1 ml reaction medium (medium B) containing mannitol (300 mM), KCl (30 mM), $MgCl_2$ (5 mM), KH_2PO_4 (2 mM), BSA (1 g litre^{-1}) and Hepes (pH 7·2; 10 mM). State 3 was elicited by adding adenosine 5′-diphosphate (ADP; 1 mM), and uncoupled respiration by adding carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP; 1 μM).

The effects of herbicides on oxidative phosphorylation were estimated by determination of ADP/O ratios. Adenosine 5′-triphosphate (ATP) synthesis was assayed by a HPLC procedure.[19]

The trans-membrane electric potential ($\Delta\psi$) was monitored following the fluorescence signal of safranine O.[20] All experiments were carried out in glass cuvettes of 1 cm light path, at 25°C, in 1·5 ml of medium B, containing safranine (10 μM), mitochondrial protein (0·3 mg ml^{-1}) and succinate (10 mM), plus the additions indicated in the legends of the Figures. Fluorescence changes were monitored with a Hitachi model F-4010 spectrofluorometer (Hitachi, Japan) at 586 nm (band pass 5 nm) with excitation at 495 nm (band pass 5 nm).

2.3 Mitochondrial swelling

Mitochondrial osmotic swelling[21] was monitored by detecting turbidity or pseudoabsorbance at 520 nm on a SLM DW2000 spectrophotometer (SLM-Aminco, Urbana, IL USA). Media were 44% iso-osmolar (100% = 270 mosmolar), containing K acetate (54 mM) or KCl (54 mM) plus Hepes (5 mM), Tris-EGTA (0·1 mM), and Tris-EDTA, (pH 7·1; 0·2 mM). The cuvette contained 2·5 ml of the reaction medium, with protein (0·5–0·75 mg), propranolol (200 μM), atractyloside (10 μM) and antimycin A (1 μM), or as indicated in the legends.

2.4 Assays of ATPase activity

The assays were performed at 25°C, in a medium containing mannitol (0·3 M), KCl (30 mM), $MgCl_2$ (5 mM), K_2HPO_4 (0·5 mM), and Hepes (pH 7·5; 2 mM). All the experiments contained mitochondria (0·2 mg protein ml^{-1}) incubated for 4 min with Triton X-100 (0·25–0·4 ml $litre^{-1}$) and trypsin (20 μg ml^{-1}).[22] The ATPase activity was calculated from the rates of released H^+ in the first minute of reaction, after addition of 2 mM ATP following a stabilization period of 15 s.

2.5 Treatment of the data

Results are presented as percentage of the controls from at least three independent experiments. Since DNOC and DNP were dissolved in ethanol, controls were carried out with addition of this solvent.

2.6 Chemicals

All reagents were of analytical grade for research. The herbicide DNOC was purchased from Fluka Chemica-Biochemica and the other xenobiotic compounds were from Sigma.

3 RESULTS

3.1 Herbicide effects on mitochondrial respiration

Succinate was commonly used as the respiratory substrate. Mitochondrial reaction media usually contain 1 g $litre^{-1}$ BSA, which is essential for satisfactory energy coupling.[23,24] Therefore, we investigated the interaction of BSA with the herbicide effects. BSA (3 g $litre^{-1}$) almost completely (85%) prevented the dissipation of the trans-membrane electrical potential elicited by 40 μM DNOC (Fig. 2A), similarly to DNP. Additionally, BSA protected against uncoupling by FCCP. This uncoupler was usually used at 1 μM to elicit the total dissipation of the trans-membrane electrical potential ($\Delta\psi$), in the absence of BSA. However, in the presence of 3 g $litre^{-1}$ BSA, an excess of FCCP (4–6 μM) was required to fully dissipate $\Delta\psi$ (Fig. 2B).

The effectiveness of a fixed amount of DNOC decreased with increasing mitochondrial protein concentration when membrane potentials and rates of oxygen consumption were assayed (results not shown). Since the results are affected by the protein concentration, the assays were always performed with similar mitochondrial concentration (*c.* 0·3 mg ml^{-1}) and BSA (1 g $litre^{-1}$), to permit comparison of data. Preparations of isolated mitochondria exhibited good membrane integrity (90%) evaluated by the outer mitochondrial membrane integrity test and the succinate-dependent respiratory indexes ADP/O and RCR were 1·6–2·0 and 3–5, respectively.[12]

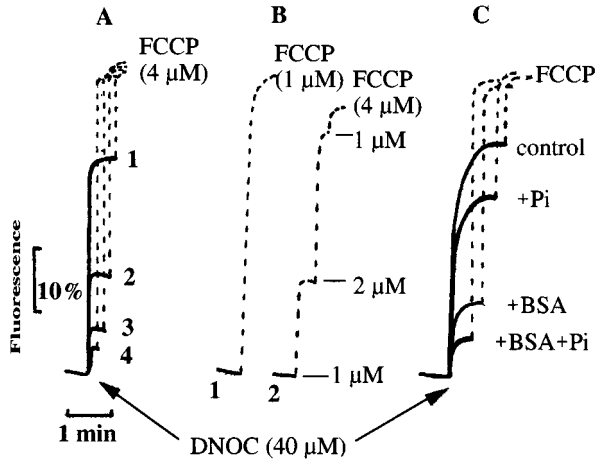

Fig. 2. Factors affecting DNOC and FCCP effects on the trans-membrane electrical potential ($\Delta\psi$) in potato tuber mitochondria, as measured by safranine O fluorescence. A, in the presence of BSA: (1) 0, (2) 1·0, (3) 2·0 and (4) 3·0 g $litre^{-1}$. B, several concentrations of FCCP used in the presence of (1) 0 and (2) 3 g $litre^{-1}$ BSA. C, in the presence or absence of 1 mM K_2HPO_4(Pi) and 1 g $litre^{-1}$ BSA, or both, as indicated. DNOC concentration is 40 μM. Total collapse of membrane potential was obtained by adding FCCP (4 μM) at the end of each assay.

A concentration of 40 μM DNOC dissipated $\Delta\psi$ more effectively in the absence of phosphate than in its presence (1 mM K_2HPO_4) (Fig. 2C). This could be observed with and without BSA (1 g $litre^{-1}$) in the reaction medium. However, there was a concurrent small decrease in the respiratory rate, suggesting that the absence of phosphate may have affected succinate transport, as the transport of carboxylic acids can be phosphate-dependent.[25]

DNOC uncouples energized mitochondria, depending on the herbicide concentration (Fig. 3A). The $\Delta\psi$ dissipation was already apparent in the presence of 10 μM DNOC, and full dissipation was obtained at about 80 μM. Similar effects were observed with DNP, but this classical uncoupler was less potent than DNOC (Fig. 3B).

3.2 Effects of DNOC on oxidative phosphorylation

The influence of DNOC on oxidative phosphorylation was tested using succinate as the respiratory substrate. At 20 μM DNOC, the value of 1·6 for ADP/O was not significantly altered (Fig. 4). However, 40 μM DNOC decreased ADP/O below 0·7. ATP synthesis was inhibited by about 10% and inhibition of more than 50% was observed only at 80 μM (Fig. 4). The sharp decrease of ADP/O was not accompanied by a sharp depression of ATP synthesis, since more than 40% synthesis was still observed at 80 μM DNOC, a concentration at

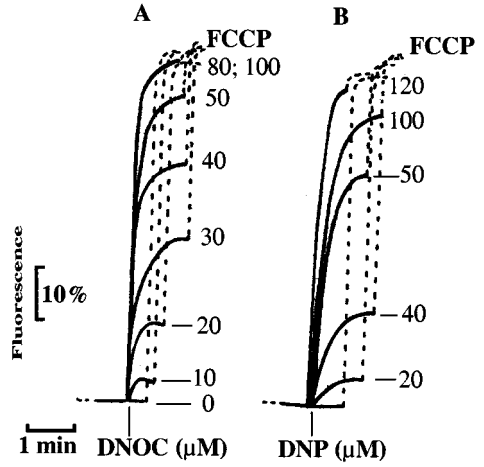

Fig. 3. Effect of DNOC and DNP on the potato tuber mitochondrial membrane potential. Mitochondria (0·3 mg m^{-1}) were added to the reaction medium as described in Section 2. The herbicide DNOC (A) and the uncoupler DNP (B) were added at the indicated concentrations (μM). FCCP (1 μM) was added at the end of each assay to elicit complete collapse of membrane potential.

which ADP/O was already non-measurable. When DNOC was replaced by FCCP (1 μM), a similar level of ATP synthesis (>40%) was recorded (Table 1). These apparently contradictory results are explained by the presence of adenylate kinase activity in the mitochondrial inter-membrane space, as previously identified for rat liver mitochondria[25] and plant mitochondria.[26] Therefore, when oxidative phosphorylation is impaired by the presence of DNOC or FCCP, in the presence of high ADP concentrations (1 mM), ATP is synthesized by the adenylate kinase activity ($2ADP \Leftrightarrow ATP + AMP$). This was confirmed by using 100 μM p1, p5-di(adenosine-5′) pentaphosphate (Ap5A), a specific inhibitor of adenylate kinase. Data from HPLC analysis indicated that ATP and AMP were produced from $ADP + K_2HPO_4$ only in the absence of Ap5A, when oxidative phosphorylation was impaired by DNOC or

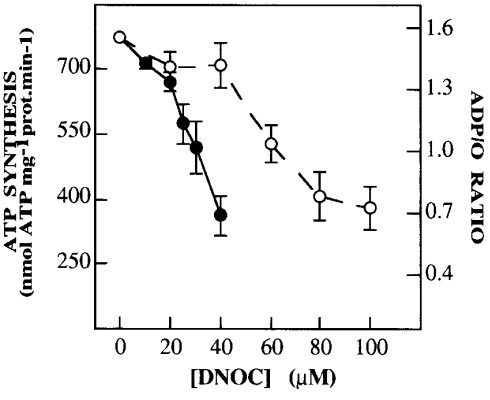

Fig. 4. Effects of DNOC on the oxidative phosphorylation of potato tuber mitochondria supported by succinate. (○) synthesis of ATP (●) ADP/O ratio. Control values of succinate-supported respiration: 786 (±97) nmol ATP mg^{-1} protein min^{-1}; ADP/O = 1·55 (±0·1). Assays were performed in the conditions described in Section 2.

TABLE 1
Effects of DNOC, FCCP and Ap5A on ATP and AMP Synthesis by Potato Tuber Mitochondria[a]

Inhibitors	*ATP synthesized (nmol ATP mg^{-1} min^{-1})*	*AMP synthesized (nmol AMP mg^{-1} min^{-1})*
0	1162 (±207)	489 (±22)
DNOC	547 (±23)	447 (±60)
FCCP	515 (±24)	524 (±38)
0 + Ap5A	783 (±158)	126 (±22)
DNOC + Ap5A	275 (±47)	17 (±30)
FCCP + Ap5A	279 (±25)	124 (±64)

[a] Determined by HPLC, in the presence of DNOC (100 μM), FCCP, (1 μM) and Ap5A (100 μM), where indicated.

FCCP (Table 1). It is unlikely that adenylate kinase was present as a contaminant, as the isolated potato tuber mitochondria had a high degree of purity and intactness as a result of efficient Percoll gradient fractionation.[12]

3.3 Effects of the herbicide on succinate oxidation

State 4 respiration of potato tuber mitochondria supported by succinate was stimulated 3-fold by 80 μM DNOC (Fig. 5A), which completely collapsed $\Delta\psi$. Above 100μM, DNOC inhibited the respiratory rate, as a function of concentration, up to about 100% at 1 mM.

The effects of DNOC on state 3 and FCCP-uncoupled respiration (Fig. 5B) were similar, except that there was a small stimulation (<10%) of state 3 at low DNOC concentrations (<100 μM). This may be related to the uncoupling effect of DNOC that simultaneously depresses oxidative phosphorylation and releases respiration from a coupled condition. The overall effect is a

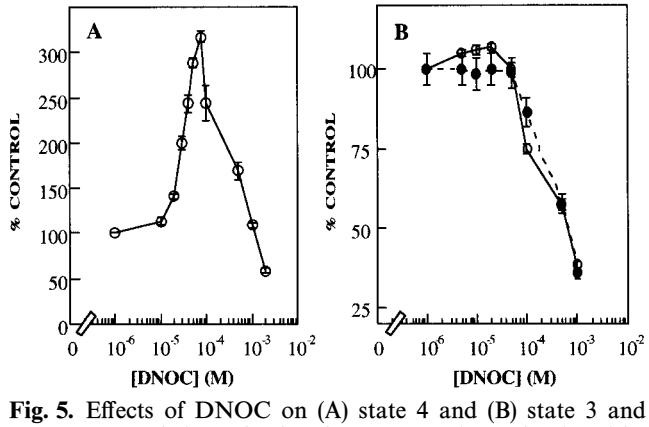

Fig. 5. Effects of DNOC on (A) state 4 and (B) state 3 and FCCP-uncoupled respiration in potato tuber mitochondria. Control values for succinate-supported respiration are expressed in nmol O_2 mg^{-1} protein min^{-1}. (A) state 4: 79 (±18). (B) state 3 (○): 190 (±33); uncoupled respiration (●): 196 (±28). 1 μM FCCP was used in uncoupled respiration assays.

small increase in oxygen consumption. This uncoupling effect of DNOC occurred in state 3 of liver mitochondria (Table 2), with an increase of more than 60% at low DNOC. This probably reflects a stronger energy coupling of state 3 in rat liver mitochondria than in potato tuber mitochondria.

The effects of DNOC and DNP on state 4 respiration exhibited similar profiles (Fig. 6A) with the following differences: low DNOC was more efficient in stimulating oxygen consumption, with maximal stimulation at about 80 μM, whereas DNP efficiency was maximal at about 150 μM; in the range from 0·2 to 1 mM, DNOC exerted stronger inhibition than DNP (Fig. 6A). The effects of the two compounds were significantly different ($P < 0{\cdot}05\%$ in Student's *t*-test for concentrations higher than 0·15 mM). The low-concentration effects of DNOC and DNP (Figs 3 and 6A) demonstrated that the greater efficiency of DNOC in stimulating oxygen consumption may be related to its capacity to dissipate the $\Delta\psi$. The high-concentration inhibitory effects of DNOC and DNP also demonstrated the increased toxicity of DNOC over DNP by experiments carried out for state 3 and FCCP-uncoupled respiration (Figs 6B and C).

3.4 Proton-dependent mitochondrial swelling

Potassium acetate (44% iso-osmolar) medium was used to assay valinomycin-induced mitochondrial swelling in de-energized mitochondria. Acetate permeates the membrane only in the neutral protonated state (acetic acid), being dissociated inside the mitochondria to produce an immediate proton gradient which impairs the entry and accumulation of the anion. Mitochondrial swelling, in the presence of 1 g litre^{-1} BSA, is very limited (Fig. 7, trace 1), as it prevents normal dissipation of the proton gradient through the uncoupling protein.[23,24] The maximal valinomycin-dependent swelling was observed on addition of FCCP (1 μM), which dissipated the proton gradient. Thus, this kind of swelling can only

TABLE 2
Effects of DNOC on Respiration Supported by Succinate in Mitochondria of Rat Liver and Potato Tubers[a]

DNOC (μM)	State 4		State 3		FCCP-uncoupled	
	Rat	Potato	Rat	Potato	Rat	Potato
0	100	100	100	100	100	100
25	—	—	150	107	—	—
50	728	288	162	99	91	99
80	725	317	—	—	—	—
100	705	244	144	75	70	86
500	288	169	73	57	29	58
1000	173	109	46	39	17	36

[a] Values represent percentage of control. Data are representative of experiments carried out with at least three different isolations of each mitochondrial type.

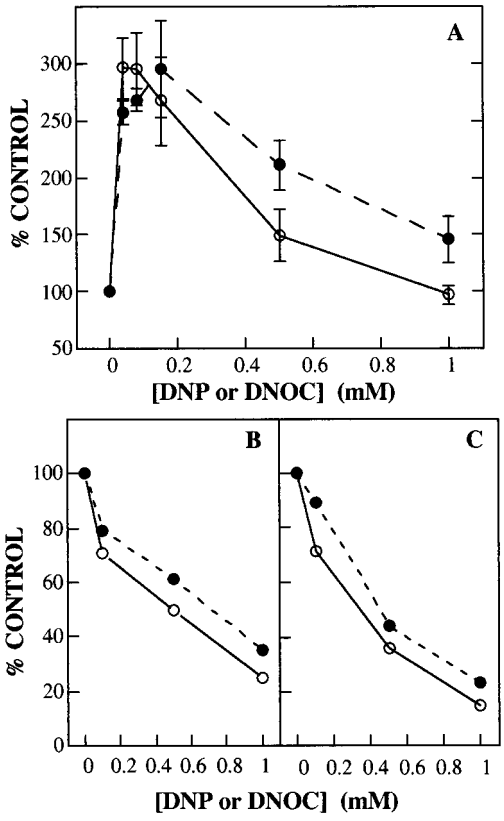

Fig. 6. Effects of DNOC as compared to DNP acting on the respiration of potato tuber mitochondria. (A) state 4. (B) state 3. (C) FCCP-uncoupled respiration. (○) with DNOC; (●) with DNP. Control value for state 4 succinate respiration: 70 (±12)nmol O_2 mg^{-1} protein min^{-1}; state 3: 212 (±30); uncoupled respiration: 215 (±26). 1 μM FCCP was used in uncoupled respiration assays. For (A), statistical significance was determined using Student's *t*-test. ($P < 0{\cdot}05$ for DNOC concentrations higher than 0·15 mM). For (B) and (C), the depicted curves are representative of a group of three independent experiments.

proceed in conditions allowing protons to exchange with K^+ entry. Therefore, swelling promoted by DNOC is a consequence of the protonophore activity of the herbicide.

The plant uncoupling mitochondrial protein, the ADP/ATP carrier, and the plant inner mitochondrial anion channel, which are potential sites of ion transport, are not involved in the effects described above, as indicated by the following: similar results were obtained in the absence and in the presence of BSA (1 g litre^{-1}) that inhibits the fatty-acid dependent uncoupling by the plant uncoupling protein;[23,24], atractyloside (10 μM) was present to inhibit the ADP/ATP carrier; and added propranolol (200 μM) prevented anion transport through the anion channel.[21]

Similar experiments were carried out in KCl medium. Atractyloside and BSA were added to avoid any contribution of the ADP/ATP carrier and the uncoupling protein. As opposed to the acetate medium, in the KCl medium, valinomycin alone induced swelling (Fig. 7B) that depended on Cl^- transport by the anion channel,

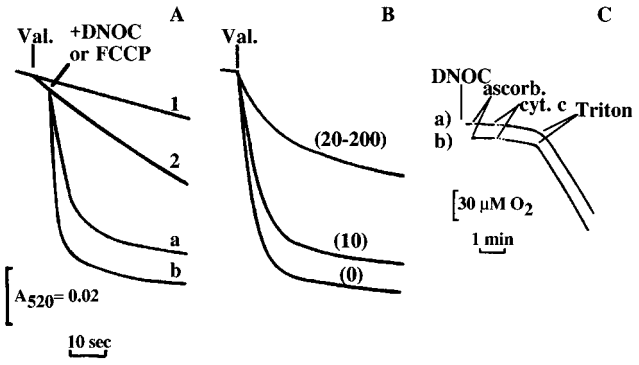

Fig. 7. Effect of DNOC on the mitochondrial swelling. (A) Swelling in 44% iso-osmolar potassium acetate (pH 7·1) supplemented with antimycin (1 μM), atractyloside (10 μM) and propranolol (0·3 mM), with (1) BSA (1 g litre^{-1}) present or (2) absent; curve (a) describes the effect of DNOC (100 μM) and (b) the effect of 1 μM FCCP; Val. indicates the addition of 1 μM valinomycin. (B) Swelling in 44% iso-osmolar KCl medium (pH 7·1) supplemented with antimycin (1 μM), atractyloside (10 μM) and BSA (1 g litre^{-1}); numbers within parentheses state DNOC concentrations (μM); the curve for 20–200 μM DNOC also describes the effect of 1 μM FCCP. (C) experiment designed to rule out putative detergent effects of DNOC; respiration supported by 15 mM ascorbate and exogenous cytochrome c (30 μM) (a) in the presence and (b) absence of DNOC (1 mM); as indicated, Triton X-100 (0·4 ml litre^{-1}) was added to disrupt the membranes, allowing the interaction of exogenous cytochrome c with the oxidase. Depicted curves are representative of a group of at least three independent experiments.

since the presence of propranolol (200 μM) completely abolished the process. The anion channel is responsible for Cl^- uptake into potato tuber mitochondria,[21] permitting an increase of KCl concentration in the matrix on valinomycin addition, allowing osmotic swelling.

DNOC inhibited valinomycin-induced swelling as a function of concentration (Fig. 7B). Concentrations of DNOC up to 20 μM maximally inhibited swelling by 55%, similarly to 1 μM FCCP. These inhibitory effects are related to the protonophoric activity of DNOC, as exchange of matrix protons with K^+ ions partially prevented accumulation of Cl^- ions in the matrix, thereby inhibiting osmotic swelling. This swelling is also a good test to evaluate the capacity of DNOC to act as a detergent. If this was the case, high DNOC could fully abolish swelling. The data in Fig. 7C rule out any detergent effect of DNOC, as at high concentrations (up to 1 mM), the respiration rate supported by ascorbate (15 mM) and exogenous cytochrome c (30 μM) was very limited and similar to that observed in the absence of DNOC. However, a strong stimulation was obtained by adding 0·4 ml litre^{-1} Triton X-100, either in the presence or the absence of DNOC. Therefore, the Triton effect (detergent) is a consequence of the permeabilization (disintegration) of the outer membrane, which allows interaction of exogenous cytochrome c with cytochrome c oxidase of the inner membrane.[12,27] Since this stimulation is not obtained in the presence of DNOC (and absence of Triton), it is safe to conclude that the herbicide, even at high concentration, has no detergent properties.

3.5 DNOC effects on the oxidation of different substrates

No significantly different effects were detected for low concentrations of DNOC (<80 μM) on the respiration rate of FCCP-uncoupled mitochondria, when different substrates were used (Fig. 8). This suggests that low concentrations of the herbicide do not interfere with the oxidative mechanism specific for each substrate. Higher concentrations of DNOC (⩾100 μM) differentially affected the respiration rate, depending on the nature of the respiratory substrate used. The respiration rate supported by malate (+pyruvate + NAD^+) was severely (75%) inhibited at 1 mM DNOC, as compared with succinate-supported respiration (58%). Furthermore, respiration supported by exogenous NADH was inhibited by only 25%, and no inhibition was observed for respiration promoted by ascorbate/TMPD, which is linked to cytochrome c oxidase.[27]

3.6 Effect of DNOC on mitochondrial ATPase activity

Activity of ATPase cannot be demonstrated in intact mitochondria isolated from potato tuber, since the inhibitory subunit of the ATP-synthase is strongly bound.[28] Triton X-100 plus trypsin, in concentrations predetermined as effective, were added to the reaction medium, to release the inhibition.[23] We usually used 0·2 mg ml^{-1} of mitochondrial protein, and obtained optimal ATPase activity in the presence of 0·25–0·4 ml litre^{-1} Triton X-100 and 10 μg ml^{-1} trypsin.

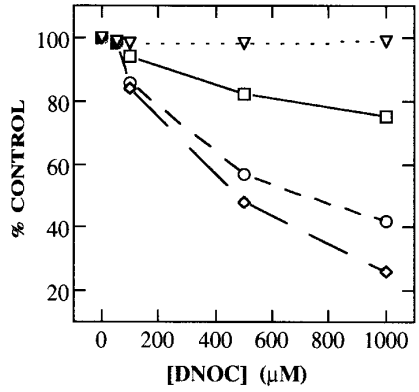

Fig. 8. Effects of DNOC on FCCP-uncoupled respiration for (◇) malate, (○) succinate, (□) exogenous NADH and (▽) ascorbate/TMPD supported respiration in potato tuber mitochondria. Assay media: malate, 20 mM malate + 4 mM pyruvate + 1 mM TPP + 1 mM NAD^+; succinate, 10 mM succinate; exogenous NADH, 1 mM NADH; ascorbate/TMPD, 10 mM ascorbate + 0·75 mM TMPD. Control values expressed in nmol O_2 mg^{-1} protein min^{-1}: malate, 116 (±10); succinate, 196 (±28); exogenous NADH, 160 (±28); ascorbate/TMPD, 300 (±41).

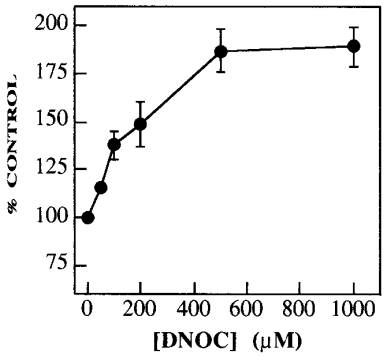

Fig. 9. Effects of DNOC on the ATPase activity of potato tuber mitochondria. The assays were performed in the presence of Triton X-100 (0·4 ml litre^{-1}) and trypsin (20 μg ml^{-1}). The assays were performed according to the conditions described in Section 2.

The ATPase activity was not significantly affected by DNOC concentrations up to 100 μM (Fig. 9). Therefore, concentrations of DNOC that promote full uncoupling of energized potato tuber mitochondria do not directly affect the ATPase complex. ATPase activity was maximally stimulated (>180%) by 500 μM DNOC (Fig. 9), showing that ATPase stimulation was obtained by DNOC concentrations that significantly inhibited respiration supported by succinate. This effect of DNOC, not related to its protonophoric activity, may be mediated by a putative effect on the conformation of ATPase catalytic subunit(s) or any regulatory subunit(s). Of interest also is the potentiating effect of low concentrations of Triton X-100 upon the state 4 inhibition by 0·5 mM DNOC, which jumps from 37 to 69% at a detergent concentration of 0·1 g litre^{-1}. Apparently the detergent renders the affected redox complexes more accessible to DNOC.

3.7 DNOC effects on animal and plant mitochondria

Some of the described effects of DNOC on potato tuber mitochondria were also investigated in rat liver mitochondria to compare the effects on corresponding mechanisms of plant and animal mitochondrial energetics. In general, similar effects of DNOC were observed for liver and plant mitochondria, although quantitative differences were detected. Increasing DNOC concentrations significantly stimulated state 3 liver mitochondria respiration (>60%) before the inhibitory effect at high DNOC (Table 2), when the effect, in plant mitochondria, was a mere 10% or less (Fig. 5B). Higher levels of stimulation, by low concentrations of DNOC (<100 μM), were also detected for state 4 respiration of animal mitochondria (maximum of >700%), as compared to the results obtained for plant mitochondria (maximum of about 350%). This difference is recorded for similar values of ADP/O and RCR, suggesting that the state 3 respiratory rate of animal mitochondria is significantly lower than the respiratory rate of uncoupled mitochondria. A different situation occurs for plant mitochondria, since, in both states, the respiratory rates are similar.

DNOC effects were also studied in FCCP-uncoupled liver mitochondria respiration (Table 2). In the presence of 1 mM DNOC, succinate-supported respiration is inhibited by about 83% of the control, whereas the corresponding plant mitochondria respiration (Fig. 5B) was less inhibited (64% of control). Functional differences or variations in the sensitivity of animal and plant mitochondria to DNOC may explain the different quantitative data.

4 DISCUSSION

The herbicide DNOC (80–100 μM), completely collapses $\Delta\psi$ (Fig. 3), thus leading to inhibition of oxidative phosphorylation of potato tuber mitochondria. Simultaneously, maximal stimulation of state 4 respiration occurs (Fig. 5A), to the same extent as induced by typical uncouplers (DNP and FCCP). In the same concentration range, the herbicide also induces maximal respiratory stimulation. Higher concentrations of DNOC (0·1 to 1 mM) strongly inhibit electron transfer supported by substrates transported to the mitochondrial matrix. In contrast, respiration supported by ascorbate (+TMPD) is not affected by DNOC, indicating that cytochrome c oxidase activity is insensitive to the herbicide.[27]

Valinomycin-induced swelling in a KCl medium was maximally inhibited at DNOC concentrations (20 μM) significantly lower than those required for maximal uncoupling in energized mitochondria (80 μM). The effect of DNOC on valinomycin-induced swelling in a KCl medium is a consequence of its action as a protonophore, resulting from an exchange of K^+ with H^+, decreasing the electrical K^+ gradient which drives Cl^- entry, thereby inhibiting mitochondrial swelling. As a consequence of a similar effect of DNOC as a protonophore, valinomycin-induced swelling in a potassium acetate medium is affected in the opposite way to swelling in a KCl medium. In this case, the protonophoric activity supports the transport of protonated acetate by membrane passive diffusion. Again, the maximal stimulation was achieved by a concentration (20 μM DNOC) significantly lower than that required for full stimulation of state 4 respiration (about 80 μM). These data demonstrate that DNOC permeabilizes the de-energized (1 μM antimycin A) mitochondrial membrane to protons by acting as a protonophore.[17,29]

Considering that inhibition of succinate-supported respiration (58%) by DNOC is higher than of that supported by exogenous NADH (25%), and that they have common electron-transfer pathways, it is tempting to

conclude that the herbicide may act on substrate transport.[30] This hypothesis is reinforced by the fact that respiration supported by malate, a substrate transported into the mitochondria, is also strongly inhibited. Moreover, the FCCP-uncoupled respiration supported by succinate and exogenous NADH are similarly inhibited by 2,4-D,[11] a herbicide with chemical similarities to DNOC, which maximally stimulates state 4 respiration of potato tuber mitochondria, before inhibition of the respiratory chain,[11] as in the case of DNOC. The diverse results also suggest that succinate dehydrogenase may be subjected to a stronger inhibition by DNOC than exogenous NADH dehydrogenase, which is at variance with the effect of the herbicide on succinate transport. This is difficult to probe by enzymatic assays, since, in plant mitochondria, three different NADH dehydrogenases are present.[31]

It is surprising to note that, in spite of the ADP/O depression by DNOC (Fig. 4) concomitant with the $\Delta\psi$ dissipation (Fig. 3), significant ATP synthesis ($>40\%$) is retained after complete dissipation of $\Delta\psi$ and depression of ADP/O by DNOC or even in the presence of FCCP. This synthesis can be assigned to adenylate kinase,[25,26] since AMP increases by a similar amount to that detected for ATP (Table 1). The decrease of ATP in presence of Ap5A, a specific inhibitor of adenylate kinase, additionally indicates that ATP is synthesized in uncoupled mitochondria by adenylate kinase putatively located in the intermembrane space of plant purified mitochondria.[26]

The loss of the DNOC methyl group converts it into the classical uncoupler DNP, resulting in a significant reduction of toxicity.[16,32] The $-CH_3$ side chain increases the efficiency of DNOC, reducing the concentrations required to achieve maximal uncoupling and inhibitory effects (Figs 3 and 6).

DNOC at low concentrations (80–100 μM), behaves as a typical uncoupler, acting like the classical uncouplers DNP and FCCP. Higher concentrations of DNOC simultaneously uncouple and inhibit respiration, behaving as an uncoupling inhibitor.[28] However, we favour the idea that DNOC can more correctly be considered as a typical uncoupler, since the effect of inhibition of respiration can only be achieved by concentrations higher than those which totally uncouple mitochondrial respiration.

Pullman *et al.*[33] refer to a stimulatory effect of DNP on the ATPase activity of beef heart mitochondria, after disruption. Since the effects of DNOC on plant mitochondria are very similar to those of DNP, we assume that the compounds act similarly on the ATPase.

Qualitatively, the effects of DNOC are similar in plant and animal mitochondria. However, some subtle differences are detected. Considering the differences of DNOC inhibitory effects recorded in animal and plant mitochondria, the results suggest that, in the case of rat liver mitochondria, state 3 respiration is normally in a higher coupling state, than with plant mitochondria. It is also apparent that the respiratory chain of liver mitochondria is more sensitive to DNOC inhibition than in plant mitochondria.

In conclusion, in plant and animal mitochondria, DNOC clearly acts as an uncoupler and protonophore at low concentrations, and as an electron-transfer inhibitor at high concentrations. This analogue of DNP acts similarly to the classical uncoupler, but it is more potent in its toxic effects on mitochondrial bioenergics.

ACKNOWLEDGEMENTS

The authors thank Luis Henrique G. Ribeiro and Matheus P. C. Vercesi for the preparation of mitochondria and excellent technical assistance.

This work was supported by the grants PBIC/C/BIO 1985/95 and Praxis/2/2.1/BIO/1156/95) of Portugal, and by PADCT, Proc. 620123/94-0 of Brazil.

This work was also supported by Convénio JNICT (Junta Nacional de Investigação Científica of Portugal—Proc. no. 423/FAPESP)/FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo of Brazil—Proc. no. 1996/0739-9). Joaquim A. F. Vicente was Visiting Professor in the Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas, 13084-970 Campinas, São Paulo, Brazil.

REFERENCES

1. Yamamoto, T., Anno, M. & Sato, T., Effects of paraquat on mitochondria of rat skeletal muscle. *Comp. Biochem. Physiol. C*., **86** (1987) 375–8.
2. Zottini, M., Scoccianti, V. & Zannoni, D., Effects of 3,5-dibromo-4-hydroxybenzonitrile (bromoxynil) on Bioenergetics of higher plant mitochondria (*Pisum sativum*). *Plant Physiol*., **106** (1994) 1483–8.
3. Zychlinski, L. & Zolnierowicz, S., Comparison of uncoupling activities of chlorophenoxy herbicides in rat liver mitochondria. *Toxicol. Lett*., **52** (1990) 25–34.
4. Steffann, S., Joly, J.-P., Loubinoux, B. & Dizengremel, P., Effects of alkylammonium on oxidative and phosphorylative properties of mitochondria isolated from *Agaricus bisporus*, *Pestic. Biochem. Physiol*., **32** (1988) 38–45.
5. Wang, G. Y., Hirai, K. & Shimada, H., Mitochondrial breakage induced by the herbicide paraquat in cultured human lung cells. *J. Electron Microsc. Tokyo*, **41** (1992) 181–4.
6. Palmeira, C. M., Moreno, A. J. M. & Madeira, V. M. C., Interactions of herbicides 2,4-D and dinoseb with liver mitochondrial bioenergetics. *Toxicol. Appl. Pharmac*., **127** (1994) 50–7.
7. Palmeira, C. M., Moreno, A. J. M. & Madeira, V. M. C., Mitochondrial bioenergetics is affected by the herbicide paraquat. *Biochim. Biophys. Acta*, **1229** (1995) 187–92.
8. Matlib, M. A., Kirkwood, R. C. & Smith, J. E., Differential effects of selected phenoxy-acid compounds on oxida-

tive phosphorylation of mitochondria from *Vicia faba* L. *J. Exp. Bot*., **23** (1972) 886–98.
9. Mannella, C. A. & Bonner, W. D. Jr, 2,4-Dichlorophenoxyacetic acid inhibits the outer membrane NADH dehydrogenase of plant mitochondria. *Plant Physiol.* **62** (1978) 468–9.
10. Moreland, D. E. & Novitzki, W. P., Effects of inhibitors and herbicides on the membrane potencial of mung bean vitochondria. *Pestic. Biochem. Physiol*., **31** (1988) 247–60.
11. Pireaux, J.-C., Dizengremel, P. & Botton, B., Effects of pesticides on the oxidative phosphorylation and electron transfer in potato tuber mitochondria. *Plant Sci*., **83** (1992) 23–30.
12. Neuburger, M., Journet, E.-P., Bligny, R., Carde, J.-P. & Douce, R., Purification of plant mitochondria by isopycnic centrifugation in density gradients of Percoll. *Arch. Biochem. Biophys*., **217** (1982) 312–23.
13. Moreland, D. E., Effects of herbicides on respiration. In *Weed Physiology*, Vol. II, ed. S. O. Duke. CRC Press, Boca Raton, FL, 1987, p. 56.
14. Braunbeck, T. & Völkl, A., Induction of biotransformation in the liver of eel (*Anguilla anguilla* L.) by sublethal exposure to dinitro-*o*-cresol: An ultrastructural and biochemical study. *Ecotoxicol. Environ. Saf*., **19** (1991) 355–74.
15. Moreland, D. E., Research on biochemistry of herbicides: An historical overview. *Z. Naturforsch*., **48c** (1993) 121–31.
16. Cremlyn. J., *Agrochemicals. Preparation and Mode of Action.* John Wiley & Sons Ltd, Chichester, West Sussex, England, 1991, p. 219.
17. Slater, E. C. Application of inhibitors and uncouplers for a study of oxidative phosphorylation. In *Methods in Enzymology* Vol. 10, ed. R. W. Estabrook & M. E. Pullman. Academic Press, New York, 1970, p. 48.
18. Bradford, M. M., A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem*., **72** (1976) 248–64.
19. Socchi, V., Cucchiarini, L., Chiarantini, L., Palma, P. & Crescenti, G., Simultaneous extraction and reverse-phase high-performance liquid chromatographic determination of adenine and pyridine nucleotides in human red blood cells. *Anal. Biochem*., **146** (1985) 118–24.
20. Holden, M. J. & Sze, H., Effects of *Helminthosporium maydis* Race T Toxin on electron transport in susceptible corn mitochondria and prevention of toxin actions by dicyclohexylcarbodiimide. *Plant Physiol*., **91** (1989) 1296–302.
21. Beavis, A. D. & Vercesi, A. E., Anion uniport in plant mitochondria is mediated by a Mg^{2+}-insensitive inner membrane anion channel. *J. Biol. Chem*., **267** (1992) 3079–87.
22. Jung, D. W. & Laties, G. G., Tripsyn-induced ATPase activity in potato mitochondria, *Plant Physiol*., **57** (1976) 583–8.
23. Vercesi, A., Martins, I. S., Silva, M. A. P., Leite, H. M. F., Cuccovia, I. M. & Chaimovich, H., Pumping plants. *Nature* (*London*), **375** (1995) 24.
24. Jezëk, P., Costa, A. D. T. & Vercesi, A. E., Evidence for anion-translocating plant uncoupling mitochondrial protein in potato mitochondria. *J. Biol. Chem*., **271** (1996) 32743–8.
25. Laterveer, F. A., Nicolay, K. & Gellerich, F. N., ADP delivery from adenylate kinase in the mitochondrial intermembrane space to oxidative phosphorylation increases in the presence of macromolecules. *FEBS Lett*., **386** (1996) 255–9.
26. Attucci, S., Carde, J. P., Raymond, P., Saint-Gés, V., Spiteri, A. & Pradet, A., Oxidative phosphorylation of mitochondria extracted from dry sunflower seeds. *Plant Physiol*., **95** (1990) 390–8.
27. Tyler, D. D., Estabrook, R. W. & Sanadi, D. R., Studies of oxidative phosphorylation and energy-linked cytochrome b reduction with tetramethyl-*p*-phenylene diamide as substrate. *Arch. Biochem. Biophys*., **114** (1966) 239–51.
28. Horstman, L. & Racker, E., Interaction between mitochondrial adenosine triphosphatase inhibitor and mitochondrial adenosine triphosphatase. *J. Biol. Chem*., **245** (1970) 1336–44.
29. Moreland, D. E., Mechanisms of action of herbicides. *Ann. Rev. Plant. Physiol*., **31** (1980) 597–638.
30. LaNoue, K. F. & Schoolworth, A. C., Metabolite Transport in mitochondria. *Annu. Rev. Biochem*., **48** (1979) 871–922.
31. Möller, I. M. & Lin, W., Membrane-bound NAD(P)H dehydrogenases in higher plant cells. *Ann. Rev. Plant Physiol*., **37** (1986) 309–34.
32. Hassall, K. A., *The Biochemistry & Uses of Pesticides*, 2nd edn. McMillan Press Ltd, London, 1990, pp. 80–1.
33. Pullman, M. E., Penefsky, H. S., Datta, A. & Racker, E., Purification and properties of soluble dinitrophenol-stimulated ATPase. *J. Biol. Chem*., **235** (1960) 3322–9.